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The Industrial Labour Welfare Association Ltd.

Bacteriological Research Laboratory

STAINS & STAINING METHODS

'GOPAL BAGH" COIMBATORE

The Industrial Labour Welfare Association Ltd.,
Bacteriological Research Laboratory,
GOPAL BAGH, COIMBATORE.

BACTERIAL STAINS.

All bacterial stains are aniline dyes of coal-tar synthetic products. They are called either acid or basic dyes; they are the salts of free colour acids or bases. The basic aniline dyes are used, to stain bacteria; they have the same staining affinities, as the chromatin, in the nucleus of tissue cells; the acid dyes have an affinity, for the cytoplasm of a cell; as the nuclear material of bacterial cells seems to be distributed, throughout their bodies, the basic dyes are employed.

Bacterial stains should bear the certification label, of the Commission on Standardization of Biological Stains. Reliable and certified manufacturers are – Coleman & Bell, Difco Laboratories, Hartman-Leddon Co., MacAndrews and Forbes, National Aniline and Chemical Co.

Certified stains are –

Alizarin red S
Aniline blue, water soluble
Auramine
Azure A
Bismarck brown Y
Brilliant cresyl blue
Brilliant green
Malachite green
Martius yellow
Methyl green
Methyl orange
Methyl violet 2B
Methylene blue (chloride)
Methylene blue thiocyanate

Carmine
Chlorazol black E
Congo red
Cresyl violet
Crystal violet
Eosin, bluish
Eosin, yellowish
Erythrosin B
Ethyl eosin
Fast green FCF
Fuchsin, acid
Fuchsin, basic
Giemsa stain
Hematoxylin
Indigo carmine
Janus green B
Jenner's stain
Light Green
Leishman's stain
Methyl violet
Neutral red
Nigrosin
Nile blue A
Orange G
Orange II
Phloxine
Pyronin
Pesazurin
Rose bengal
Safranin O
Sudan III
Sudan IV
Tetrachrome Stain (MacNeal)
Thionin
Toluidine blue O
Wright stain.

SOLUBILITY OF SOME DYES IN WATER and ALCOHOL.

Name of dye	Percent in 100 cc. of Water at 26°C.	Grams soluble in 100 cc. of 95 per cent Alcohol.
Bismarck brown Y	1.36	1.08
Bismarck brown R	1.10	0.98
Chrysoidin Y	0.86	2.21
Chrysoidin R	0.23	0.99
Congo red	....	0.19
Crystal violet (chloride)	1.68	13.87
Eosin Y (Na salt)	44.20	2.18
Erythrosin (Na salt)	11.10	1.87
Fuchsin, basic, new	1.13	3.20

Name of dye	Percent in 100 cc. of Water at 26°C.	Grams soluble in 100 cc. of 95 per cent Alcohol.
Fuchsin, basic, rosaniline chloride	0.39	8.16
para-rosoniline chloride	0.26	5.93
para-rosaniline acetate	4.15	13.63
Fuchsin, acid	....	....
Gentian violet (see methyl and crystal violet)	....	....
Janus green	5.18	1.12
Malachite green (oxalate)	7.60	7 52
Methyl violet	2.93	15.21
Methylene blue (chloride)	3.55	1.48
Neutral red (chloride)	5.64	2.45
Picric acid	1.18	8.96
Pyronin B (iodide)	0.07	1.08
Pyronin G	8 96	0.60
Rosanilin	0.39	8.16
Para-Rosanilin	0.26	5.93
Rose bengal (Na salt)	36.25	7.53
Safranin	5.45	3.41
Sudan III	0.00	0.15
Thionin	0.25	0.25
Toluidin blue O	3.82	0.57

All dyes should be weighed accurately on an analytical balance.

Dyes should be ground in a mortar with the diluent, adding a small amount of the diluent at a time until all has been added.

All staining solutions should be filtered before use. Staining solutions when prepared should be

allowed to stand for at least 24 hours, before they are used.

No solution should be used after precipitation has occurred; when precipitation occurs, discard the solutions from the bottom of the bottle, after the rest of the stain has been used. It is convenient to keep saturated alcoholic solutions of dye powders, and from these to make up aqueous solutions, by adding ten times the amount of distilled water and filtering before use.

Diluted stains should be made, in small quantities. Stock solutions keep for a much longer period of time, particularly those containing either carbolic acid or potassium hydroxide. All staining solutions should show on their labels, the concentration of the dye present and the date of manufacture.

Keep all staining solutions, out of direct sunlight, and in ground glass-stoppered bottles.

One per cent aqueous solutions of dyes, are called *simple stains*, and these are capable of staining, many of the bacteria in films from cultures, body fluids and tissues. *Differential stains* are used to stain bacteria; they not only reveal the structure of bacteria, but also show very marked differences, in their staining affinities. Differential stains are thus very helpful, in the identification of organisms. The two most important differential staining methods are Gram's method, by means of which all bacteria are grouped either as Gram-positive or as Gram-negative; and the Ziehl-Neelsen's method groups them, as either acid-fast or nonacid-fast.

Mordants are chemicals added to simple staining solutions, to give the dye greater penetrative powers, and also to fix it in the bacterial protoplasm, so much so, that when the decolourising agents are applied to the film or tissue, to bleach the ground work in which the bacteria lie, the bacteria themselves will retain the stain. Heat may be applied during staining, with the same object, in view. Examples of mordants are, carbolic acid in carbol-fuchsin, potassium hydrate in Lœffler's alkaline methylene blue, etc. Examples of decolourising agents are, mineral acids as in Ziehl-Neelsen's method, chloroform, clove oil or aniline oil in Claudius's method, acid-alcohol for tubercle bacillus. The spores, capsules and flagella of bacteria, require special staining methods, to demonstrate them.

METHODS EMPLOYED FOR THE ROUTINE STUDY OF BACTERIA.

As bacteria consist of clear protoplasmic matter, differing but slightly in refractive index, from the medium in which they are grown, it is difficult, with the ordinary microscope except when special methods of illumination are used, to see them in the unstained condition. The morphology of the different bacteria, therefore, is best studied by means of stained smears. Their protoplasm reacts to stains somewhat similar to the nuclear material of tissue cells. By staining, their morphology and identification, becomes possible.

For protozoa in films of blood, etc., *compound* stains are used, known as Leishman's, Wright's, Giemsa's and Jenner's stain. All these are

modfications of the original Romanowsky compound stain. These stains also stain many bacteria. The hyphomycetes do not as a rule require staining, in order to demonstrate and study them. The saccharomycetes stain by some of the more intense methods, used for bacteria.

As some of the bacteria are motile, it is best to begin with a study of the motility of the organism.

Hanging-drop Method.

To make a hanging-drop preparation, take a loopful of a young (4 to 8 hour growth) broth culture, or a loopful of salt solution into which is emulsified, a small amount of growth from an agar slant; place it in the centre of a thin clean cover-glass. Ring with vaseline round the cell of a clean hollow-ground glass slide, the coverslip is inverted over the concavity of the hollow-ground slide; the coverslip is so inverted over the concavity that the drop of bacterial suspension hangs from the coverslip without touching the slide. The drop must not touch the vaseline ring. If a hollow-ground slide is not available, the coverslip may be ringed thickly and inverted over an ordinary slide.

In the microscopic examination, the concave mirror of the microscope should be used. The light coming through the diaphragm of the microscope is reduced. Using the low-power objective, the edge of the drop is located. Then the high-power (dry) objective is moved into place, and the light readjusted. After focusing on the edge of the drop, the slide is moved about, and the bacteria observed for motility.

The oil-immersion objective can be used, if a drop of thin oil is placed on the cover-slip, but this is not advisable; neither is it necessary, as a routine procedure.

Hanging-drop preparation must be sterilized before cleaning. During examination, great care must be taken, not to break the coverslip, because of the danger, of contaminating the lens and stage of the microscope.

Preparation of films:

Films are made from blood, exudates and transudates; from cultures (liquid and solid) of micro-organisms, and from tissue juices, and tissues.

It is very important that great care should be exercised to make good, even films, for staining. Results of microscopic examination are solely and entirely dependant on how good the smear is.

Blood films:

Prepare three smears on perfectly clean grease-free and polished slides.

Place slides in glacial acetic acid for 15 minutes; wash in distilled water; place in 95% alcohol for 5 minutes; dry with clean handkerchief; pass through flame and cool before using.

Touch the end of a slide to a large drop of blood; then spread the drop with a second slide placing the edge across the drop and allowing it to spread to the entire length of the edge; holding the second slide at an angle of 45°, spread the drop evenly. As soon as

the blood has spread entirely, across the spreader slide held at an angle of about 45°, with a rather quick movement, push and do not pull the spreader towards the other end of the under slide. If made too slowly, the spread will be too thin. Allow the blood to dry, in the air. It is important to hold the spreader at a proper angle.

Methods of staining blood smears:

An examination of the red blood cells and different kinds of leucocytes possesses a great deal of diagnostic value, in addition to the detection of any micro-organisms, that may be present.

As already stated, the blood must be prepared in thin, evenly distributed smears on slides. Well-prepared films or smears are absolutely essential for accurate results. For routine work, a variety of staining methods are available preference should be given to polychrome stains, that are capable of staining the neutral, acid and basic granules of leucocytes. For this, Wright's method, Leishman's method (which is the same in procedure as Wright's), Jenner's method, Giemsa's method and Pappenheim's method are available.

Preparation of films of bacteria:

For the examination of bacteria of cultures from a solid medium, a small loopful of distilled water or saline is placed on a clean glass slide, a colony or growth is touched with the tip of a platinum needle, and a minute amount of the growth is stirred into the loopful of water, so as to give a faint turbidity. The mistake of adding too much of the bacterial

growth is very frequently made. This should be avoided. The drop is spread into a thin film and allowed to dry in air. With a fluid medium a drop is spread on the slide without dilution, and allowed to dry in air.

The smear is fixed by passing the slide, film side up, through a flame four or five times. Or the film may be flooded with absolute ethyl or methyl alcohol, or glacial acetic acid, and after one or two minutes washed with water and dried in air. Films to be examined for tubercle bacilli, should be fixed a second time in alcohol after heat fixation, but as a rule, heat fixation is adequate. The film should be allowed to cool before staining.

For routine work, it is recommended that films be stained with Lœffler's alkaline methylene blue and by Gram's method. Ziehl-Neelsen's carbol-fuchsin freshly diluted with water (1 in 10) may be substituted for Lœffler's methylene blue.

Liquid petrolatum is by far the best mounting medium. It is always of proper consistency, and is never sticky. Unlike canada balsam, it does not develop any acidity, and permanent mounts retain their colour, for at least two years. This is particularly important, with preparations of blood, malarial parasites, trypanosomes, intestinal flagellates, etc., in which a well-stained chromatin is necessary, for identification. It is also useful for mounting small insects and sporangia of moulds. Furthermore, it has superior optical properties. For permanent preparations, the border of the coverglass should be sealed with some cementing material.

Coverslips: These should be $\frac{3}{4}$ or $\frac{7}{8}$ inch square and of No. 1 thickness. They are cleaned by placing them in a mixture of nitric acid 6 parts, potassium bichromate 6 parts, water 100 parts. They should be dropped one by one into the fluid in the solution contained in an evaporating dish and boiled. Coverslips are then well washed first in tap water, then in distilled water, and stored in a stoppered jar in 50% alcohol. Before use, they are dried with a soft clean cloth such as an old handkerchief.

GENERAL STAINS.

Preparation of staining solutions:

Stock solutions consist of saturated alcoholic or aqueous solutions of dyes.

a. Saturated alcoholic solutions:

Basic fuchsin – 3 g. in 100 cc. (95%) alcohol
Methylene blue – 7 g. in 100 cc. (95%) alcohol
Gentian violet – 4.8 g. in 100 cc. (95%) alcohol

b. Saturated aqueous solutions:

Methylene blue – 6.7 g. in 100 cc. of distilled water
Gentian violet – 1.5 g. in 100 cc. of distilled water

In preparing saturated solutions, it is best to employ an excess of the commercial dye (at least 50% excess) to insure saturation, and filter the solution before use.

Saturated solutions are not efficient for staining; they must be adequately diluted. Diluted stains should be made in small quantities. Stock solutions keep for a much longer period of time, particularly those containing carbolic acid or potassium hydroxide.

All solutions should show on their labels the concentration of the dye present at the date of manufacture.

Keep all staining solutions out of direct sunlight in ground-glass – stoppered bottles.

Preparation of Stock Solutions.

a. *Saturated alcoholic soluion of Gentian violet:*

Gentian violet	5 g.
95% alcohol	100 cc.

Grind the gentian violet in mortar, adding alcohol slowly, until the dye is in solution. Place in a glass-stoppered bottle and filter through filter paper after 24 hours.

b. *Saturated alcoholic solution of Basic fuchsin:*

Basic fuchsin	3.5 g.
95% alcohol	100 cc.

Carry out the technique above.

c. *Saturated alcoholic solution of Methylene blue:*

Methylene blue	1.5 g.
95% alcohol	100 cc.

Carry out the technique above

d. *Lugol's solution:*

This solution is 15 times the strength of Gram's iodine solution, and may be used as a stock, from which Gram's iodine is made.

Iodine resublimed	5.0 g.
Potassium iodide, C. P.	10.0 g.
Distilled water	100.0 cc.

Rub up the iodine and potassium iodide in a mortar until they are throughly mixed. Add water, a small amount at a time, and grind it into the mixture, until all the water has been added. Allow to stand in a glass-stoppered bottle, and filter after 24 hours.

e. *Ten percent solution of Potassium hydroxide:*

Dissolve 10 g. of potassium hydroxide, C. P. sticks in about 50 cc. distilled water in a stoppered Pyrex flask; cool under running water if the flask becomes hot; dilute to 100 cc. with distilled water.

f. *Five percent solution of Carbolic acid:*

Made from carbolic acid crystals or liquid carbolic acid. Liquid carbolic acid is made by adding 4 cc. distilled water to every ounce of melted carbolic acid crystals.

If the crystals are used, they are melted, and 5 cc. of the melted carbolic acid are used in 100 cc. solution.

If the liquid carbolic acid is used, 5.74 cc. of the liquid carbolic acid are used in 100 cc. solution.

Technique: Place 50 cc. distilled water in a 100 cc. graduated cylinder; add either 5 cc. liquefied carbolic acid crystals or 5.74 cc liquid carbolic acid; dilute to 100 cc. with distilled water.

g. *One percent solution of carbolic acid:*

This is made from the 5% solution by diluting one part of 5% carbolic acid solution to a total of 5 parts using distilled water. If made from the

melted crystals, 1 cc. of the liquefied carbolic acid crystals is added to 50 cc. distilled water in a 100 cc. graduated cylinder, and then diluted to 100 cc. with distilled water.

DILUTED STAINS.

a. *Carbol Gentian violet*:

Made from the stock saturated alcoholic solution.

Add 10 cc. saturated alcoholic solution of gentian violet to 50 cc. of 1% carbolic acid solution in a 100 cc. graduated cylinder; dilute to 100 cc. with 1% carbolic acid solution. Filter after 24 hours.

If made direct from Gentian violet crystals:

Gentian violet	1 g.
Carbolic acid crystals	2 g.
Absolute alcohol	10 cc.
Distilled water to make	100 cc.

Rub up the gentian violet crystals and the alcohol in mortar; add the carbolic acid crystals and mix; add ⅔rds of the water, a small amount at a time, stirring constantly; pour the mixture into a bottle, rinse the mortar with the remaining water, adding it to the mixture in the bottle. Filter through filter paper after 24 hours.

b. *Carbol fuchsin*:

Made from the stock saturated alcoholic solution.

Add 10 cc. saturated alcoholic solution of basic fuchsin to 50 cc. of 5% carbolic acid solution in a 100 cc. graduated cylinder. Dilute to 100 cc. with 5% carbolic acid solution. Filter after 24 hours.

Made direct from basic fuchsin crystals:

Basic fuchsin	1 g.
Carbolic acid crystals	5 g.
Absolute alcohol	10 cc.
Distilled water to make	100 cc.

Proceed as above for carbol gentian violet,

c. *Carbol methylene blue*:

Methylene blue	1.5 g.
Phenol	5.0 g.
Distilled water	100 cc.
Dissolve and mix with	
Ethyl alcohol	10.0 cc.

d. *Carbol thionin (Nicolle)*:

Thionin	1.0 g.
Phenol	2.5 g.
Distilled water	100 cc.

Filter; and dilute before use with equal quantity of distilled water.

e. *Loeffler's methylene blue*:

Distilled water	100 cc.
10% solution of potassium hydroxide	0.1 cc.

(this gives a concentration of potassium hydroxide of 1 : 10,000)

Saturated alcoholic solution of methylene blue	30 cc.

Filter after 24 hours.

f. *Gram's iodine*:

Made from the stock Lugol's solution.

Add 10 cc Lugol's solution to about 100 cc. distilled water in a graduated cylinder; dilute to

150 cc. with distilled water and mix. Filter after 24 hours.

Made direct from iodine crystals –

Iodine, resublimed	1 g.
Potssium iodide, C. P.	2 g.
Distilled water	300 cc.

Proceed as for Lugol's solution above.

g. Acetic thionin (Frost)

Thionin	1 g.
Distilled water (hot)	1200 cc.
Dissolve and filter; then add	
Glacial acetic acid	60 cc.

This staining solution is recommended for staining bacteria in agar films inoculated with milk. Not ordinarily used in pure culture work.

h. Archibald's thionin-methylene blue:

Solution A.		*Solution B.*	
Thionin (90% dye content)	0.5 g.	Methylene blue (90% dye content	0.5 g.
Phenol	2.5 g.	Phenol	2 5 g.
Formalin (40%)	1.0 cc.	Formalin (40%)	1.0 cc.
Water	100 cc.	Water	100 cc.

Allow each solution to stand for 24 hours. For use, mix in equal parts and filter. Stain fixed films for 10 seconds.

i. Pappenheim's pyronin-methyl green:

Methyl green (50% dye content)	1.0 g.
Pyronin (Commis. certified)	0.25 g.
Phenol	2.0 g.
Ethyl alcohol (95%)	5.0 g.
Glycerin	20.0 cc.
Distilled water	100 cc.

Dissolve the dye in alcohol and phenol and pyronin in the mixture of glycerin and water. Mix the two solutions and filter. Employed for the demonstration of bacteria in pus and other organic liquids.

j. Dorner's Nigrosin solution:

Nigrosin (B Grubler) or	
American nigrosin certified	10 g.
Distilled water	100 cc.
Boil 30 minutes in an Elenmeyer flask; then add as a preservative	
Formalin (40%)	0.5 cc.

Filter twice through double filter paper and put in serological test tubes, about 5 cc. to the tube.

This staining solution is used for the negative demonstration of bacteria, in place of the Burri Indian Ink. A loopful of the bacterial suspension is mixed on the slide with the same quantity of negrosin solution. After drying, the slide can be examined without a cover glass.

k. Gabbett stain:

Methylene blue	2.0 g.
25% sulphuric acid	100 cc.

l. Aniline Gentian violet:

Solution A.	
Crystal violet (85% dye content)	2.5 g.
Ethyl alcohol (95%)	12.0 cc.
Solution B.	
Anilin	2.0 cc.
Distilled water	98.0 cc.

Shake and allow to stand for a few minutes; then filter. Mix solutions A and B. This does not keep long; should be freshly mixed.

or,

Add 10 cc. aniline oil to 100 cc. distilled water, shake throughly for about 15 minutes; filter through filter paper; to three parts of this aniline water, add 1 part of saturated alcoholic solution of gentian violet. Filter through paper after mixing.

Stabilized Gentian violet (Kilduffe):

Solution A.

Formalin	5 cc.
Distilled water	95 cc.

Solution B.

Saturated alcoholic solution of Gentian violet. Mix 25 cc. of solution B with 75 cc. of solution A.

The following staining solutions are frequently used as *contrast or counter stains.*

(i) Eosine:

Yellowish (water soluble) eosine	1.0 g.
Distilled water	100 cc.
Dissolve and then add Alcohol (95%)	5 cc.

(ii) Safranin:

Safranin	0.5 g.
Distilled water	100 cc.

(iii) Bismarck brown (vesuvin):

Bismarck brown	0.5 g.
Distilled water	100 cc.

(*iv*) *Chrysoidin*:

Chrysoidin	1 g.
Distilled water	300 cc.

(*v*) *Dilute carbol-fuchsin*:

Carbol fuchsin	10.0 cc.
Distilled water	100.0 cc.

(or 1 : 14 dilution)

(*vi*) *Picric acid*:

Saturated solution of picric acid in distilled water.

(*vii*) *Malachite green*:

Malachite green	0.5 g.
Distilled water	100 cc.

(*viii*) *Neutral red*:

Neutral red	1.0 g.
1% aqueous acetic acid solution	2.0 cc.
Distilled water	1000 cc.

(*ix*) *Crystal violet*:

Crystal violet	0.5 g.
Distilled water	100.0 cc.

(*x*) *Methyl violet*:

Methyl violet (6B)	0.5 g.
Distilled water	100 cc.

(*xi*) *Methylene blue*:

Methylene blue, saturated alcoholic solution	5.0 cc.
Distilled water	95.0 cc.

(*xii*) *Gentian violet*:

Gentian violet, saturated alcoholic solution	5.0 cc.
Distilled water	95.0 cc.

SPECIAL STAINS.

GRAM STAIN -- SOLUTION 1.

Carbol Gentian violet (Nicolle)

Solution A.

Crystal violet (85% dye content)	2 g.
Ethyl alcohol (95%)	10 cc.

Solution B.

Phenol	1 g.
Distilled water	100 cc.

Mix solutions A and B.

GRAM STAIN – SOLUTION 2.

Lugol's solution

Iodine	1 g.
Potassium iodide	2 g.
Water (distilled)	300 cc.

Stain for 1 to 2 minutes with Gentian violet, then for 1 to 2 minutes with Lugol's solution. Decolourise with 95% alcohol until no more colour is removed. Counterstain with safranin; or decolourise with alcohol-acetone, and counterstain with eosine or safranin.

Hucker's Modification of Gram stain (S. A. B.)

Solution A.

Crystal violet (85% dye content)	4 g.
Ethyl alcohol	20 cc.

Solution B.

Ammonium oxalate	0.8 g.
Water (distilled)	80.0 cc.

Mix solutions A and B in equal volumes; if this mixture is too difficult to decolourise, dilute solution A as much as ten times, if necessary.

Counterstain:

Safranin (2.5% in 95% alcohol)	10.0 cc.
Water	100.0 cc.

Stain 1 minute with ammonium oxalate-crystal violet solution; immerse in Lugol's iodine solution for one minute, wash in water and blot dry; decolourise in 95% ethyl alcohol for 30 seconds, wash, dry and examine.

Kopeloff and Beerman's Modification of *Gram's stain* (S. A. B.)

Solution A.

Gentian or crystal violet	1 g.
Water (distilled)	100 cc.

Solution B.

Sodium bicarbonate	1 g.
Water (distilled)	20 cc.

Just before use, mix 30 drops of solution A with 8 drops of solution B.

Iodine solution:

Iodine	2 g.
Sodium hydroxide	10 cc.

After the iodine is dissolved, make up to 100 cc. with water.

Counterstain:

Basic fuchsin	0.1 g.
Water (distilled)	10.0 cc.

Stain 5 minutes or more with alkaline gentian violet; rinse with iodine solution, then add more iodine solution and allow to stand two minutes or longer. Drain off iodine solution and blot dry (without washing).

Decolourise with 50% acetone in alcohol by dropping on to slide and permitting it to drain off until no colour is seen to come off (generally less than 10 seconds), then dry in air. Counterstain 10–30 seconds; wash in water, dry and examine.

GRAM'S STAIN (Lillie's modification)

Solution A.

Crystal violet	2 g.
Ethyl alcohol (95%)	20 cc.
Ammonium oxalate (1%) in distilled water	80 cc

Solution B.

Potassium iodide	2 g.
Crystal iodine	1 g.
Distilled water	300 cc.

Solution C.

Acetone

Solution D.

Safranin	1 g.
Distilled water	100 cc.

Solution A, B and D are prepared by mixing their respective ingredients and filtering. Solution A keeps well.

The fixed smear is covered with solution A for 30 seconds; then rinsed in water; flooded with solution B for 30 seconds; rinsed in water; and decolourised with acetone (solution C).

Note: Hucker uses 70 cc. of 95% ethyl alcohol + 30 cc. acetone for decolourisation.

Following decolourisation, flood the smear with solution D for 10 seconds. Wash in water, dry and examine

Gram's method of staining (modified):

Prepare the film thin and evenly;
Fix in flame
Pour on Gentian violet on slide by filtering it.
Leave for 5 minutes
Pour off stain
Pour Gram's iodine solution
Leave for 1 minute
Pour off this and pour in fresh Gram's solution
Leave for 1 minute
Decolourise it with acetone (cheaper, quicker and cleaner) till no more colour comes off the slide
Wash with water
Counterstain with either dilute carbol fuchsin or safranin.

Gram's iodine solution (modified)

Iodine	1 g.
Potassium iodide	2 g.
Distilled water	240 cc.

When this is dissolved, add 60 cc. of 5% aqueous solution of sodium bicarbonate. This solution is better than ordinary Gram's iodine solution which, when exposed to light and heat, may become acid and so decolourise normally Gram positive bacteria.

For counterstaining, may use dilute carbol fuchsin 1 in 20, or ½% solution of watersoluble eosin in distilled water.

GRAM-STAINING REACTIONS of IMPORTANT BACTERIA.

Gram-negative.	*Gram-positive:*
Cocci	Cocci
Meningococcus	Staphylococcus group
N. catarrhalis	Streptococcus group
Gonococcus	Pneumococcus group
	G. tetragena
Bacilli	Bacilli
K. Pneumoniæ	Corynebacterium group
P. æruginosa	C. tetani
F. dentium	M. tuberculosis
E. coli group	M. lepræ
Salmonella group	B. anthracis
Shigella group	C. welchii
M. Mallei	Other spore-forming anærobes
C. chauvœii	
P. tularensis	
P. pestis	
H. influenzæ	
H. pertussis	
H. ducreti	
H. lacunatus	
H. conjunctivitidis	
Brucella group	
Spirilla	Spirilla
V. cholera and allied forms	None important.
Mouth spirals	

As Gram's Method is an important staining method, the following points must be constantly kept in mind.

(a) Laboratory cultures and subcultures carried over for years frequently lose their Gram characteristics.

(b) Cultures which are several days old, or dead or degenerated, do not stain characteristically.

(c) Iodine solution deteriorates and becomes light in colour; it should be of a rich portwine shade.

(d) Decolourisation is best observed over a white background and should be stopped as soon as no more violet stain is seen to stream out from the preparation. Decolourisation can be controlled by making a preparation of a known Gram-positive and a known Gram-negative organism on either side of the smear to be stained.

(e) The preparation of smear for staining should be thin and evenly spread.

ACID-FAST BACILLI.

Stains for Mycobacterium Tuberculosis.

(a) Ziehl-Gabbett method.

Solution 1.

Preparation of Gabbett's methylene blue:

Methylene blue (dry)	2.0 g.
Sulphuric acid (sp. 1.84)	25.0 cc.
Distilled water	75.0 cc.

Solution 2.

Carbol fuchsin:

Basic fuchsin (saturated alcoholic solution)	10.0 cc.
Phenol (5%)	100.0 cc.

Shake the mixture and allow to stand several days, shaking occasionally and filter.

The smear is stained with hot carbol fuchsin for 4 or 5 minutes and counterstained with Gabbett's methylene blue for 30 seconds. The tubercle bacilli stain red; other bacteria in the field, blue.

(b) Schulte-Tiggs method.

(i) Flood the slide with carbol fuchsin.

(ii) Heat the slide to steaming and continue heating for one minute (avoid excessive heating).

(iii) Wash with tap water, and decolourise with sodium sulphite (10% Na_2SO_3 in water which should be prepared fresh, at least weekly).

(iv) Wash and counterstain with picric acid (saturated aqueous solution).

(v) Wash in water, dry and examine.

The tubercle bacilli are stained red, other bacteria yellow.

ZIEHL-NEELSEN'S METHOD:

Method for staining acid-fast bacilli.

Solution A.

Basic fuchsin	1 g.
Ethyl alcohol	10 cc.
Phenol (5%) aqueous solution	90 cc.

Solution B.

Hydrochloric acid (concentrated)	3 cc.
Ethyl alcohol	97 cc.

Solution C.

Lœffler's alkaline methylene blue.

Fixed smear is covered with carbolfuchsin and steamed gently over a flame for 3 minutes. Stain is renewed repeatedly to *prevent drying* on the slide. (Carbolfuchsin may be allowed to act without steaming for 30 minutes or overnight at room temperature). Five minutes will suffice for the leprosy bacillus. After staining with carbolfuchsin, wash the smear in water; decolourise with acid alcohol until thin areas are colorless; again wash with water; counterstain with Lœffler's alkaline methylene blue, for 30 to 60 seconds; wash with water, dry.

To avoid overstaining, 1 : 10 dilution of Lœffler's stain is used just long enough to give the background a faint-blue colour.

In place of solutions B and C, 1 : 4 dilution of Gabbett's solution (2 gm. methylene blue, 100 cc. of 25% sulphuric acid) can be used. After staining with solution A, wash the slide in water, and immerse it in diluted Gabbett's solution for 5 minutes or longer if the film shows any residual redness. Then wash in water and dry. Stained by the above method, acid-fast organism appear as red rods against a blue background. Leprosy bacilli often are decolourised more readily than tubercle bacilli and if staining for leprosy bacilli, decolourisation with acid alcohol should be light. In such cases, use a 5 to 20% aqueous solution of sulphuric acid. Smegma bacilli also are usually more easily decolorised than tubercle bacilli.

Pappenheim's Method of Differentiation.

After steaming the smear in carbolfuchsin, the stain is poured off and without washing, the film is treated with the following: Corallin 1 gm; absolute alcohol 100 cc; methylene blue 0.66 gm; and glycerin 20 cc. The film is flooded several times with the mixture and allowed to drain off slowly following each application.

Then wash in water and mount. The smegma bacilli is decolourised by the corallin and alcohol but the tubercle bacilli remain red.

FONTE'S METHOD: to demonstrate Much granules.

Stain the preparation with carbolfuchsin, decolourise with acid-alcohol, then carry through the various steps of Gram's method, counterstaining with Bismark brown; by this method the acid-fast tubercle bacilli show as red rods dotted with violet granules. Those which do not fully retain acid-fast properties show a zigzag violet lines.

Decolourising Agents.

(i) Acid-alcohol:

Add 3 cc. of concentrated hydrochloric acid to enough 95% alcohol to make a total volume of 100 cc.

(ii) Absolute alcohol or 95% alcohol.

(iii) Alcohol-acetone:
equal parts of absolute alcohol and acetone.

(iv) Acetone by itself

(v) 70 cc. of 95% ethyl alcohol, +30 cc. acetone. (Hucker)

(vi) Fresh solution of sodium sulphite (10% in distilled water)

Method for Staining Diphtheria
(STAINS FOR DIPHTHERIA)

a. Loeffler's methylene blue :

Methylene blue (sat. alcoholic soln.)	30 cc.
Potassium hydroxide (1 in 10,000)	100 cc.

Prepare film in the usual way, fix and stain for 3 to 5 minutes.

Polychrome methylene blue is better for the staining of C. diphtheriæ. This alkaline methylene blue is ripened by storage for several months in half filled bottles which are shaken at intervals.

b. Neisser's Stain.

Solution 1.

Methylene blue	0.1 g.
Absolute alcohol	20.0 cc.
Glacial acetic acid	5.0 cc.
Distilled water	100.0 cc.

Stains for 10 to 30 seconds with solution 1, wash in water and counterstain with Bismarck brown or safranin for 5 to 30 seconds.

The granules stain blue, the body of the cells brown or pink.

Neisser's Diphtheria Stain.

Solution 1.

Methylene blue (dye content not specified; probably 90%)	1.0 g.
Alcohol (95%)	20.0 cc.
Acid Acetic (glacial)	50.0 cc.
Distilled water	950.0 cc.

Dissolve the dye in alcohol and add the rest.

Solution 2.

Crystal violet (dye content not specified; probably 85%)	1.0 g.
Alcohol (95%)	10.0 cc.
Distilled water	300 cc.

Solution 3.

Chrysoidin	1 or 2 g.
Hot water	300 cc.

Dried films stained 10 seconds in a mixture of 2 parts of solution I and 1 part of solution II. Wash; stain ten seconds in solution III. Wash briefly in water or *not at all;* blot dry.

Neisser's Stain for Diphtheria Bacilli.

Fix slide in flame, allow to cool.
Stain with Neisser soln. No. I 30 – 60 Sec.
Wash with distilled water
Stain with Neisser soln. No. II 5 – 15 Sec.
Wash with distilled water
Dry and examine.

The granules stain blue, the bodies of bacilli stain a light yellowish brown.

Epstein Stain for Diphtheria Bacilli.

Fix slide in flame; allow to cool.
Stain with Lœffler's methylene blue 1½ minute.
Wash with distilled water.

Pour on Gram's iodine, then blot it up immediately. Granules stain heavy blue; bodies of bacilli stain greenish.

BECK'S STAINING METHOD (for demonstrating Diphtheria bacilli)

Solution A.

Crystal violet (saturated alcoholic solution)	10 cc.
Acetic acid (4.3% aqueous solution)	90 cc.

Solution B.

Bismarck brown	0.48 cc.
Distilled water	125.00 cc.

To prepare the Bismack brown solution, the water is heated to boiling; stain is added and boiling continued for 2 minutes. The solution is filtered and cooled.

Solution A is applied for 2 minutes. Wash the slide in water and excess moisture drained off. Then stain with solution B for 30 seconds, wash and dry.

The metachromatic granules stain purple while the remainder of the cell body and most other bacteria take the brown colour. Lœffler's methylene blue can be substituted when this method of staining is not available.

Stains for C. Diphtheriae.

In its identification, always advisable to use Gram's stain since C. diphtheriae is Gram positive, a point of value in identification. This stain obscures the metachromatic granules. Lœffler's methylene blue, when well ripened, is a useful stain as it demonstrates both the morphology of the bacillus and the granules.

In addition –

Neisser's stain:

1. Neisser's methylene blue – 3 minutes.

2. Wash rapidly with water.

3. Counter stain. A great variety of counter stains are used.

a Bismarck brown – 3 minutes.

Since this stain is not intensive, it does not tend to obscure the granules when these are poorly developed.

b. Chrysoidin – 1 minute.

c. Safranin – 2 minutes.

d. Lœffler's methylene blue – 3 minutes.

The combination of the two methylene blues is used by some as the routine stain for the detection of C. diphtheriæ in cultures. It reveals both the morphology of the organism and the granules. The results are improved by treating the film with Lugol's iodine for 1 minute and washing with water between steps 2 and 3.

c. Albert's stain.

Solution 1.

Toluidin blue	0.15 g.
Methyl green (or malachite green)	0.20 g.
Glacial acetic acid	1.0 cc.
Alcohol (95%)	2.0 cc.
Distilled water	100 cc.

After standing for one day, the solution is filtered and is ready for use.

Solution 2.

Iodine	2.0 g.
Potassium iodide	3.0 g.
Distilled water	300 cc.

The solution is ready for use as soon as the iodine is entirely dissolved.

Stain with solution 1 for one minutes, wash in water, dry with good absorbent filter paper; stain with solution 2 for one minute; wash and dry with filter paper. Granules of diphtheria bacilli stain black, the bars dark green and intermediate portions are light green.

Albert's method (modified)

1. Toluidin blue-malachite green, – 5 minutes.
2. Do not wash off. Blot dry.
3. Lugol's iodine, – 1 minute.
4. Do not wash off. Blot dry.

This is a good stain for granules which are stained blue-black, the bodies of the bacilli being blue.

Pugh's Stain.

Toluidin blue	2 g.
Absolute alcohol	20 cc.
Glacial acetic acid	50 cc.
Distilled water	950 cc.

Fix the smear by heat as usual; flood the film with the stain and heat to steaming; allow the stain to act for one to three minutes. Wash in water, dry and examine.

This stain is used in a particular way as follows :–

1. Place a drop of the stain on the fixed film.
2. Cover with No. 1 cover glass.
3. Examine with oil-immersion lens.

Granules are blue; the bodies of the bacilli are almost unstained.

This is a very useful and rapid single stain; the protoplasm of the bacilli is stained light blue and the granules are coloured reddish purple.

Ponder's Stain.

Toluidin blue (Grubler)	0.02 g.
Glacial acetic acid	1.00 cc.
Absolute alcohol	2.00 cc.
Distilled water to make	100.00 cc.

Make a film on a cover glass and fix it in the usual way; spread the stain on the film, turn over the cover-slip and mount as a wet preparation.

The metachromatic granules come out with great intensity and the bacillary body is stained a lighter colour.

Bie's Stain.

Methylene blue	2 g.
Dahlia	0.25 g.
Absolute alcohol	20.00 cc.
Glacial acetic acid	50.00 cc.
Distilled water	950.00 cc.

The stains are dissolved in alcohol which is then added to the mixture of acetic acid and water.

Make a film on a cover-glass and fix it in the usual way. Put a drop of the stain on the slide and mount cover-glass on it; remove excess of the stain by soaking it in blotting paper. Examine the preparation under oil immersion lens without delay.

The preparation is not permanent and fades in a short time. Diphtheria bacilli are seen with pale blue protoplasm and deep blue granules; other organisms are only feebly stained.

Diphtheria Stains.

Stain of von Staltenberg, H.

Malachite green	0.25 g.
Toluidin blue	0.05 g.
Hæmatoxylin	0.01 g.
Distilled water	100.00 cc.
Acetic acid	3.00 cc.
Absolute alcohol	3.00 cc.

Dissolve malachite green and toluidin blue in distilled water and hæmatoxilin in absolute alcohol; then add acetic acid; filter.

Fix the film in the usual way; pour the solution on the slide and allow it to act for 2 to 5 minutes. Wash in tap water, dry and examine under oil immersion lens.

The granules are stained red or purple and the bodies are green.

GUTSTEIN and NEISSER'S STAIN

(Azure II eosin)

1. Stain – 1% aqueous solution af Azure II

2. Decolourising agent – 0.5% acetic acid or aqueous solution of chrysalinine

Stain 2 to 3 minutes with 1% aqueous Azure II solution; wash in tap water; differentiate with 0.5% acetic acid for one to three seconds. Metachromatic granules are stained reddish-violet, while the bodies are stained blue.

or,

stain 2 to 3 minutes with 1% aqueous Azure II solution; wash in tap water; wash with aqueous chrysalinine. Metachromatic granules are stained black and the bodies of the bacilli are yellow.

Simple Stains for Morphology.

Loeffler's Methylene blue stain:

Fix slide in flame; flood slide with Lœffler's, and stain for $1\frac{1}{2}$ – 3 minutes; wash with distilled water; dry and examine.

This is an excellent stain for metachromatic granulations of *diphtheria bacillus*.

Dilute Carbol-fuchsin Stain:

Dilute carbol-fuchsin 1 to 10 with distilled water; fix slide in flame; flood with dilute carbol-fuchsin stain; leave it in contact with the slide for 30 to 60 seconds. Wash in distilled water; dry and examine.

This is a good stain to demonstrate morphological characteristics.

Stain for Bacterial Granules.

Ljubinsky stain –

Original formula.

Solution A.

Pyoktanin (Merck)	0.25 g.
5% acetic acid	100 cc.

Solution B.

0.1% vesuvin

Amended formula.

Solution A.

Methyl violet 2B or Crystal violet (85% dye content)	0.25 g.
Glacial acetic acid	5 cc.
Distilled water	95 cc.

Solution B.

Bismarck brown Yellow	0.1 g.
Distilled water	100 cc.

Stain smears ½ to 2 minutes in solution A; wash with water; stain with solution B ½ minute; wash, dry and examine.

Staining Spores.

a. Moller's method:

Prepare smear, fix by heat; flood the slide with chloroform for 1 to 2 minutes; wash.

Treat with 5% chromic acid solution for one minute; wash in water.

Stain with hot carbol-fuchsin for 2 to 5 minutes. Decolourise with 1% sulphuric acid for a few seconds; counterstain with Lœffler's methylene blue, ½ to 1 minute; wash in water, dry and examine. The spores will stain red, vegetative cells and sporangia and outer wall of spores should stain blue.

Moller's method. (improved)

1. Carbol fuchsin with heat – 10 minutes.
2. Wash with water.
3. Decolourise with 5% solution of sodium sulphite – ½ minute.
4. Wash with water.
5. Counterstain Lœffler's methylene blue – 1 minute
6. Wash with water.

Spores are stained bright red, protoplasm blue. The optimum time for step 3 must be determined by trial as spores of some bacteria are more easily decolourised than those of others.

An alternative method is to substitute for steps 5 to 6.

(5a) blot and dry.

(6a) Spread a thin film of nigrosin over the film with another slide.

(7a) dry rapidly.

In this modification, the red spores contrast with unstained bodies of the bacilli.

b. Schaeffer and Fulton's method.

Prepare a smear, fix by flame by passing through it three times; flood with malachite green (5% aqueous solution allowed to stand half an hour and filtered) and heat to steaming, three to four times within one half minute. Wash off excess stain under tap water for about half minute.

Apply 0.5% aqueous safranin solution for one half minute. Wash, blot, dry and examine.

c. Abbott's method.

Make smears, fix in the usual way; cover with Lœffler's methylene blue and heat until it boils; repeat the boiling at intervals, but do not boil continuously, and continuing this for one minute. Wash in distilled water; decolourise with a mixture of 98 parts of 80% alcohol and 2 parts nitric acid until all blue has disappeared. Wash in distilled water; dip from 3 to 5 seconds in mixture of 90 cc. distilled water and 10 cc. saturated alcoholic solution of eosin. Rinse in distilled water, blot dry and mount in balsam. Spores stain blue, bacilli stain pink.

d. Dorner's Spore staining method.

Solution A.

Nigrosin	10 g.
Distilled water	100 cc.
Formalin	0.5 cc.

Solution B.

Freshly prepared and *filtered* carbol fuchsin.

To make solution A, nigrosin is added to boiling water and boiling continued for 30 minutes. After cooling, the formalin which serves merely as a preservative is added. The solution is filtered twice through paper and stored in 5 cc. amounts, in ampoules.

A heavy suspension of the organism under study is made in 2 to 3 drops of distilled water in a small test tube; an equal quantity of carbolfuchsin is added; the mixture is allowed to stand in a boiling water bath for 10 to 12 minutes.

On a slide, one loopful of the stained preparation is mixed with one looplul of a saturated aqueous solution of nigrosin. This is smeared as thinly as possible and dried rapidly. The spores are stained red, the bodies of the bacteria are almost colourless and stand out against the dark-gray background of nigrosin.

Staining Capsules.

a. The Hiss Capsule Stain.

Prepare smear, fix in ordinary way; stain with aniline oil gentian violet or carbol fuchsin for five seconds, or until steam arises, when heated. Wash off the stain with 20% aqueous copper sulphate solution. Blot and dry in air; do not wash.

NOTE: Make smears in serum instead of in water; allow to dry and fix in flame or in 4% formalin. Cover with a mixture of 5 cc. saturated alcoholic solution of gentian violet and 95cc distilled

water; heat to steaming, and continue steaming for a few seconds. Wash off the dye with a 20% solution of copper sulphate; allow to dry in air and examine.

Organisms and cells stain purple; capsules are unstained or pale lavender background, cells and bacteria may all stain purple, leaving the capsules unstained and looking like halos around the bacteria.

b. The Huntoon Capsule Stain.

Solution 1. to be used as a diluent.

Three grams of sodium caseinate (nutrose) are sifted into 100 cc. of distilled water and heated to 100'C in a steamer for one hour. Add 5 cc. of 2% aqueous solution of carbolic acid to serve as a preservative. Decant into test tubes and allow to settle. Employ the supernatant fluid as the diluent. (Since the supernatant liquid tends to become thinner by constant precipitation of the nutrose, the solution should occasionally be reboiled).

Solution 2. Fixing and staining solution.

2% aqueous solution of carbolic acid		100 cc.
Concentrated lactic acid	...	0.25 to 0.5 cc.
1% acetic acid	...	1.0 cc.
Saturated alcoholic solution of basic fuchsin		1.0 cc.
Carbol fuchsin (old)	...	1.0 cc.

This solution must be kept tightly corked, and it will then keep very well.

Experience has shown that in the above solution the addition of old and fresh fuchsin as given makes a better product than an increase in the amount of either one alone.

Technique of staining –

(i) Employ the nutrose solution No. 1 as a diluent, emulsifying the bacteria in one or two loopfuls and then spreading on the slide as thin a film as possible with the loop. The use of the edge of a slide in spreading the film, as in blood work, is not to be recommended. (ii) Allow to dry in air; *do not fix with heat.* (iii) Cover the film with the fixative and staining solution No. 2 and allow to act for 30 to 45 seconds. (iv) Wash quickly in water, dry and examine.

c. Gins' Capsule Stain.

Place near one end of the slide a drop of Indian ink diluted with an equal amount of water (the ordinary indian ink sold for drafting purposes usually contains too many bacteria to be satisfactory; a special grade known to be free from bacteria should be used; this bacteria-free ink is at present very difficult to obtain). Mix with it a loopful of a bacterial suspension; smear over the slide in a thin film using the edge of another slide as in making a blood film; the resulting film should appear gray rather than black. Dry in air, and fix with heat or preferably methyl alcohol. Stain with any ordinary bacterial staining fluid. The capsules appear colourless between the stained bacterial cell and the background of ink.

d. Welch's Capsule Stain.

Make smears in serum instead of in water; allow to dry; cover with glacial acetic acid for a few seconds; pour off the acid, and cover with aniline gentian violet solution.

Place *Distilled water 100cc, and aniline oil 10cc.* in a bottle and shake vigorously for 10 to 15 minutes; filter through filter paper and add 1 part of saturated alcoholic solution of gentian violet to 3 parts of aniline oil-water, and filter. Wash in 1 or 2% solution of sodium chloride. Capsules stain lavender around a purple organism.

e. Gray's Capsule Stain.

This is a modification of Muir's method. It yields more well defined capsules than the original method. It is used only on cultures, not for exudates or sections.

Prepare light suspensions of organisms in tap water and allow a loopful to dry on a cool slide, *do not spread the film.* Fix by gentle heat; place a small rectangular piece of filter paper on the slide to cover the film and over-saturate it with carbol-fuchsin; heat by passing 14 times (7 seconds) through the flame, or until steam rises; the paper keeps the stain in place on the film. When the slide is cool, or after about 30 seconds, wash off the paper and excess stain; apply Muir's mordant for 5 to 15 seconds. Muir's mordant is made as follows:—

Saturated solution of mercuric chloride	2 parts
20% tannin solution	2 parts
Saturated solution of potassium alum	5 parts

Wash well in running water; re-stain for 30 seconds with any suitable staining solution containing methyl alcohol; the following have given good results:

i.	Methyl hydrate	50 cc.
	Basic fuchsin 1% aqueous solution	50 cc.
ii.	Methyl hydrate	50 cc.
	Methylene blue 1% aqueous solution	50 cc.

iii. A mixture of No. 1 and No. 2 in the proportion of 10 : 5.

The contrast between the red cell and the blue capsule appears to be better with No. iii than with No. ii. The better definition given by this modification than by the original method appears to be due to the omission of the treatment with ethyl alcohol after the first staining operation. Gray states that the films were examined with light from a 100-watt lamp and that the capsules of some bacteria, such as Neisseria, may be seen better if a ground glass screen is used under the condenser.

Capsular method of staining.

Fix the smear with strong formalin or 10% tannic acid and then stain by Gram's method, counterstain with eosin.

Special Staining Methods.

Wayson's Staining method: for P. Pestis, P. tularensis and streptobacillus moniliformis. A good stain for *demonstrating capsule*.

Fuchsin, basic	0.2 g.
Methylene blue	0.75 g.
Phenol (99.5%)	10 cc.
Alcohol (absolute ethyl)	20 cc.
Distilled water	200 cc.

The dyes are dissolved separately in separate portions of alcohol; phenol is dissolved in water; the dissolved dyes are added to the phenolized water and the solution filtered.

The stain is applied *undiluted* for 10 to 30 seconds; then washed, dried and examined; it gives a clear pink-and-blue differentiation.

Staining Flagella.

Loeffler's method:

This method is applicable only to cultures. Use agar culture not more than 18 – 24 hours old; add enough of this culture to produce a faint cloudiness in distilled water. Place a small drop of this turbid suspension on a coverglass free of grease (to render it so, warm them in concentrated sulphuric acid, wash in water, and keep in a mixture of alcohol and strong ammonia. Dry them on a fat-free cloth;) Spread by tilting. Dry quickly; fix by heating over a flame while holding in the fingers; cover with freshly filtered mordant, and warm gently for 1 minute;

Mordant:

20% aqueous tannic acid solution.	10 cc.
Saturated solution of ferrous sulphate, cold	5 cc.
Saturated alcoholic or aqueous gentian violet	1 cc.

Wash in water; cover with freshly filtered aniline gentian violet; warm gently for one half to one minute; wash in water; dry and mount in balsam.

Van Ermengen's method:

Place the smears for 1 hour at room temperature or 5 minutes over a water-bath at 100°C in the following solution –

2% osmic acid	1 part
10 – 25% tannin	2 parts

Wash successively with water, absolute alcohol, and water; place for a few seconds in the following solution –

0.5% solution of silver nitrate in distilled water; Transfer without washing, to the following solution.

Gallic acid	5 g.
Tannin	3 g.
Fused potassium acetate	10 g.
Distilled water	350 cc.

Keep in this solution for a few seconds; place again into the 0.5% solution of silver nitrate until the film begins to turn black, wash and examine.

Weiss Method.

Mordant:

(i) 100 gm. tannic acid in 100 cc. 95% alcohol.
(ii) 7.5 cc. glacial acetic acid in 100 cc. formalin (40% Formaldehyde)

For use, mix 1 part of (i) with 2 parts of (ii)

Stain: 5 gm. gentian violet is added to 100 cc. formalin. Shake; heat slightly; allow to stand one week at room temperature before filtering.

Technique:

Add to an 18 hour broth culture enough glacial acetic acid to make a 5% solution. Place in a water bath at 37°C for 15 minutes; transfer a loopful to a clean slide, and allow it to dry. Cover with the mordant, and steam for 2 to 5 minutes; wash with warm water for one to two minutes; cover with stain for one to two minutes. To increase the intensity of the stain, treat further with a stain composed of 10 parts of acid green in a mixture of 100 parts of alcohol diluted 33⅓%, for ten minutes. Wash, dry and examine.

Shunk's Flagella Stain.

(1)Mordant:–

Solution A.

Saturated aqueous solution of tannic acid	30 cc.
Solution of ferric chloride (5% in water)	10 cc.

(by saturated solution, unless otherwise specified, is meant a solution saturated at room temperature, 21 – 25°C.)

Solution B.

Aniline	1 cc.
Ethyl alcohol (95%)	4 cc.

Solution A is best prepared a week or more ahead of time and filtered before use; when using, place about 8 drops of solution A on the slide and immediately add one drop of solution B; there is then a precipitation on the slide; the excess mordant

is carefully drained off, and the stain applied without previous washing.

(2) Stain: Carbol fuchsin, 1% safranin in 50% alcohol, aniline gentian violet, or Lœffler's methylene blue may be used. Shunk recommends the following, however:–

Lœffler's methylene blue solution	30 cc.
Solution B of Shunk's mordant	3 cc.

This solution is immediately ready for use and *keeps well.*

Casares Gil's Flagella Stain.

Mordant:–

Tannic acid	10.0 g.
Aluminium chloride (hydrated)	18.0 g.
Zinc chloride	10.0 g.
Basic fuchsin ‡	1.5 g.
Alcohol (60%)	40.0 cc.

(‡ Authors specify rosanilin hydrochloride; there are however, other basic fuchsins more universally available which ought to prove equally satisfactory)

Gray's Flagella Stain.

(1) Mordant:–

Solution A.

Potassium alum (saturated aqueous solution)	5.0 cc.
Tannic acid (20% aqueous soln.)	2.0 cc.

(a few drops of chloroform must be added to this, if a large quantity is made up).

Mercuric chloride (saturated aqueous solution)	2 cc.

Solution B.

Basic Fuchsin (saturated alcoholic solution) 0.4cc.

Mix solution A and B in less than 24 hours before using. Both solutions, separately, may be kept indefinitely, but deteriorate rapidly after mixing.

(2) Stain: Carbol-fuchsin.

General method of applying flagella stain: very young, vigorous cultures must be procured (in general, 12 – 24 hour growths on agar). Use agar slopes with plenty water of condensation; transfer a loopful of this water of condensation from 24 hour culture to second slant; then 24 hours later, pour off one or two drops of condensation water aseptically into a tube of sterile distilled water of the same temperature as the culture; incubate at optimum temperature of the organism for 48 to 72 hours. Better to leave in distilled water for only 7 hours. Cover glass or slide must be scrupulously clean. For this, place it in hot cleaning solution (Potassium bichromate solution), clean in strong sodium or potassium hydroxide, rinse in weak hydrochloric acid, and then in distilled water. Keep in alcohol until ready for use. Burn off alcohol from cover-glass just before use, in the flame. Greatly dilute the growth in distilled water and make smear. The preparation should be thin enough to dry immediately and the slide should be warm enough to allow rapid drying.

After drying, apply mordant, (filtered before use if this is called for) for ½ – 3 minutes, depending on the type of organism. With Lœffler, use just enough heat to allow visible steam to rise from the solution. Casares-Gil, Shunk, Gray mordants are used cold.

Warm stains usually require 1 minute, while 3 minutes are usually allowed for cold stains. Some recommend 5 minutes.

Poor results are due to (1) greasy cover glasses and slides; (2) old cultures; (3) unsatisfactory medium; (4) too slow drying of smears; (5) too thick suspensions of bacteria; (6) insufficient or excessive mordanting (7) understaining or overstaining. (8) Personal equation also enters into the picture.

Staining Spirochætes.

Fontana Tribondeau's Stain:

Solution A.	
Acetic acid (glacial)	1 cc.
40% Formalin	10 cc.
Distilled water	100 cc.
Solution B.	
Tannic acid	5 g.
1% phenol	100 cc.
Solution C.	
5% silver nitrate solution	50 cc

A few cc of silver nitrate solution is reserved. To the remainder is added, drop by drop, concentrated ammonia solution until a sepia precipitate, which forms, redissolves. The silver nitrate solution is shaken and stirred constantly during the addition of ammonia. Then some of the reserved silver nitrate solution is added, drop by drop, until there occurs a slight clouding, which persists on shaking. This solution will remain *useful for several months.* Occasionally, it should be poured into a clean receptacle, and if it has become clear, a few more

drops of 5% silver nitrate are added. On a clean slide, a thick film of fluid from a chancre, or other material containing spirochætes, is made and allowed to dry in the air. The film is covered with solution A for 1 minute; it is washed thoroughly with distilled water, covered with solution B, and heated until the fluid steams; it is again washed with distilled water. It is now covered with solution C and heated until the fluid steams. This is allowed to act for 30 seconds. The slide is washed with water and dried in the air or blotted. The stained film should be of a maroon color; the spirochætes stained dark brown or black.

Other Staining Methods:

(1) Thin films are fixed in absolute alcohol for 5 to 10 minutes; before the alcohol evaporates, (to prevent troublesome deposits) wash off the alcohol in distilled water; then stain with blood stain. A very good stain is equal parts of eosin (B. A. or water soluble of good quality in) 1:20,000 (aqueous) and 1:2000 polychrome mythelene blue. Keep the solutions separate and only mix just before use. Films are allowed to stain for 1 to 2 hours or overnight in a covered petri dish. Rinse in distilled water and allow to dry. Spirochætes stain purplish red and easily distinguished.

Fontana's method - modified.

Make film as thin as possible and then wash for 1 to 2 minutes in the following solution (fixative).

Pure Glacial acid	1 cc.
Formalin (40%)	2 cc.
Distilled water	100 cc.

Then wash the film in running water for ½ minute and pour on the following mordant.

Phenol (carbolic acid)	1 cc.
Tannic acid	5 g.
Distilled water	100 cc.

Warm the slide until it steams and keep for about ½ minute. Rinse with distilled water.

Pour on the ammoniacal silver nitrate solution and warm for about ½ minute and wash and dry.

(*Ammoniacal silver nitrate solution:*– to a 0.25% solution of silver nitrate in distilled water, add liquid ammonia until the precipitate which first forms is redissolved. Then add silver nitrate solution, drop by drop, until the liquid becomes just opalescent.)

By this method the spirochætes are covered with a deposit of metallic silver and stand out very distinctly as dark filaments much thicker than the unstained living spirochætes.

(2) *Indian ink method*: a good quality of Indian ink must be used; Chinchin or Gonther's are the best. A drop of the exudate from chancre is placed on a perfectly clean slide and a drop of Indian ink is placed by its side; mix the two drops and with another slide's edge may take a thin smear; allow to dry completely which may take an hour. Examine under oil immersion lens. The unstained spirochætes stand out against a black brown back ground, of the Indian ink particles.

(3) *Congo red method*: Mix thoroughly on a slide 1 or 2 loopfuls of a 2% aqueous solution of congo red and exudate; make a thin even film on the slide and allow to dry in air; when dry, wash very gently with 1% aqueous solution of hydrochloric acid. Do not use water or blotting paper afterwards, but just drain off the acid and dry the film in air. Spirochaetes are unstained and stand out white against a blue black-ground.

(4) *Collargol method*: Place 1 part of collargol in a bottle with 19 parts of distilled water; shake repeatedly to ensure solution; then on a slide, place 1 drop of the material to be examined and one drop of this collargol solution; mix and make a thin film. Allow to dry well in air; and examine under oil immersion lens. Spirochætes show on the slide as black against a colourless background.

(5) *Romanowsky's method*: After the film is dry fix it with absolute alcohol and then stain with a 1 in 2 dilution of Giemsa's stain for 1 hour; Leishman stain may also be used; precipitate should not be found on the slide. Spirochætes stain a rose-pink.

(6) *Dark-ground illumination:* This is by far the best method of examination. A special dark-ground condenser is necessary, also a small diaphragm inside it or a funnel shaped stop is placed inside the oil immersion lens. Cedar oil is placed between the slide and the condenser. A good strong light has to be used. Very careful centering of the condenser is necessary in order to get good illumination; and this is usually done first with the 2/3rd lens.

Methods of Demonstrating Spirochætes.

Some spirochætes, such as Sp. vincentii can be stained with simple stains; others such as those of the relapsing fevers can be rendered visible by Leishman's stain; but a number, require special methods.

Indian Ink or Nigrosin:

By the use of one of these, spirochætes may be rendered visible, standing out unstained against a dark background.

Fontana's Methods:

This is probably the most satisfactory method of demonstrating spirochætes in films. It is a silver impregnation method, the spirochætes being rendered visible by the deposition of metallic silver on their surfaces, which makes them appear much coarser than they really are.

1. Dry film without heat.

2. Fix in acid formalin solution for about 1 minute, the reagent being renewed three times during this period.

3. Wash with water well.

4. Treat with mordant for ½ minute, gentle heat being applied.

5. Wash well with water.

6. Treat with silver solution for ½ minute, gentle heat being applied.

7. Wash well with water.

The spirochætes are stained dark brown or black. The silver solution should not be kept for more than a few hours as, if kept for a longer time, a brown precipitate may form and this is a most violent explosive.

Levaditi's method:

This is also a silver impregnation method but since it is applied to blocks of tissue and not to individual sections, it is not here described. The treated tissue is imbedded in paraffin and sections are cut. These, when fixed to slides, merely require to be freed from paraffin wax with xylol and mounted in Canada balsam or examined in oil without a cover glass. Spirochætes appear black and tissue cells, brown. It is almost the only satisfactory method for the demonstration of spirochætes in organs removed at post-mortem examination.

Staining Rickettsiae.

Castaneda's Stain:

Solution A.

Potassium dihydrogen phosphate	1 g.
Disodium phosphate ($Na_2HPO_4.12\ H_2O$)	25 g.
Distilled water	200 cc.
Formalin	1 cc.

Each salt is dissolved in 100 cc. of water, the two solutions are mixed together, and the formalin added as a preservative. The pH of this buffer solution should be 7.5

Solution B.

Methyl alcohol	100 cc.
Methyl blue	1 g.

Solution C.

Safranin "O" (0.2%)	25 cc.
Acetic acid (0.1%)	75 cc.

To 20 cc. buffer, 1 cc. formalin and 0.15 cc. solution B are added. The slide is stained with this mixture for 3 minutes and at the end of this time, the stain poured off but the slide is *not washed;* the smear is covered with the safranin solution for 2 to 5 seconds and washed immediately in running water.

Staining Fungi.

Cotton–blue Lactophenol:

Phenol crystals	20 g.
Lactic acid	20 cc.
Glycerol	40 cc.
Distilled water	20 cc.
Cotton blue	0.05 g.

The phenol is dissolved in the liquids by heating gently; the dye is added when solution is complete.

Specimens are cleared in 10% potassium hydroxide and washed gently in water. The washed specimens are placed in a drop of the stain and covered with a coverslip. The fungus elements will be dark blue, the remainder of the structures, a light blue. Permanent mounts can be made by using glycerin as a clearing agent rather than the hydroxide and by sealing the coverslip to the slide.

TETRACHROME STAIN (*MacNeal*)

Eosin Y (dye content 80.85%)	1 g.
Methylene blue (dye content about 90%)	1 g.
Azur A	0.6 g.
Methyl violet (Bernthsen)	0.2 g.
Methyl alcohol (neutral and acetone free)	1000 cc.

Heat to 50°C, shake thoroughly and leave at 37°C for a day or two; then filter and use filtrate. This stain can now be purchased either dry or in solution.

THE ROMANOWSKY STAINS.

The original Romanowsky stain was made by dissolving in methyl alcohol the compound formed by the interaction of watery solutions of eosin and zinc-free methylene blue.

It has been replaced by various modifications and these are Leishman's, Wright's, Jenner's and Giemsa's stains. These are now available prepared in dry form for use.

The peculiar property of the Romanowsky's stains is that they impart a reddish-purple colour to the chromatin of malaria and other parasites. This colour is due to a substance which forms when methylene blue is ripened, either by age, as in polychrome methylene blue, or by heating with sodium carbonate. The ripened methylene blue is mixed with a solution of water-soluble eosin, when a precipitate due to the combination of these dyes, is formed.

This precipitate is washed with distilled water, dried and dissolved in pure methyl alcohol (pure for analysis) which has a pH of 6.5; if too acid, the reaction must be adjusted, with an indicator by the addition of N/100 NaOH. Each modification of the Romanowsky stain varies according to the ripening and the relative proportions of methylene blue and eosin. For the cytological examination of blood, Jenner's stain is used; for the malaria parasite and trypanasomes Leishman's and Wright's modification give the best results; while the pathogenic spirochætes (particularly the Treponema pallidum of syphilis) and certain protozoa can be demonstrated best by Giemsa's stain. The Romanowsky stains except Jenner's stain, are usually diluted for staining purposes with distilled water; when a precipitate is formed, it is removed by subsequent washing.

All these stains can be purchased ready for use. They may be made by dissolving the powdered stain in 100 cc. pure methyl alcohol.

For Jenner's stain – dissolve 0.5 g. of powdered stain in 100 cc. pure methyl alcohol.

Pour the stain on the dried but unfixed film and allow to act for one to three minutes; wash the film with distilled water until pink. Blot the slide and allow to dry in the air, and examine under oil immersion lens.

For Leishman's stain – Dissolve 0.15 g. of Leishman's powder in 100 cc. pure methyl alcohol. The powder is ground in a mortar with little methyl

alcohol, the residue of undissolved stain allowed to settle, and the fluid decanted into a bottle; the residue in the mortar is treated with more methyl alcohol and the process is repeated until all the stain goes into solution. The remainder of methyl alcohol is now added. This stain is improved by keeping two weeks before use.

Dry unfixed films – the stain is first used undiluted and methyl alcohol fixes the film; the stain is then diluted with distilled water and the staining proper carried out.

Pour the undiluted stain on the unfixed film and allow it to act for 2 minutes; by means of a pipette and rubber teat, add double the volume of distilled water to the slide, mixing the fluids by alternately sucking them up in the pipette and expelling them. Allow the diluted stain to act for 7 to 10 minutes. Wash the slide gently with distilled water allowing the preparation to differentiate in the distilled water until the film appears bright pink in colour – usually about a minute. Remove the excess of water with blotting paper and dry in the air. It is important that the reaction of the distilled water be neither acid nor alkaline. Any slight variation from neutrality may alter considerably the colour of the granules in white blood corpuscles, etc., and give rise to supposed pathological appearances in cells which are really normal. Keep neutral water in large aspirator bottle, specially for these stains. Add 2 or 3 drops of 1% aqueous neutral-red solution. The usual reaction of distilled water is slightly acid and a few drops of 1% sodium carbonate solution

should be added until the solution shows the faintest possible suggestion of pink colour. Much trouble will be eliminated if a buffer solution is used instead of distilled water for diluting the stain and washing the slide. It is made as follows –

Disodium hydrogen phosphate (anhydrous)	5.447 g.
Potassium dihydrogen phosphate	4.752 g.

Mix together in a mortar and keep as such; the buffer mixture is quite stable.

Add 1 g. of buffer mixture to 2000 cc. of distilled water and this gives a pH of 7.0 which is suitable for most work.

Some samples of stain may require a slightly more acid solution of pH 6. 8. For this mix,

Disodium hydrogen phosphate (anhydrous)	4.539 g.
Potassium dihydrogen phosphate	5.940 g.

Add 1 g. of the mixture to 2000 cc. of distilled water.

Note:– When staining is excessively bluish, as in old films, good differentiation is obtained by brief washing with 1% monosodium phosphate.

For demonstrating Schuffner's dots in Benign Tertian Malaria the use of Giemsa's stain following Leishman's stain has been recommended as follows –

Fix thin blood film with Leishman's stain for 15 to 60 seconds, dilute with twice the volume of buffer solution at pH 7.0 and stain for 15 minutes;

wash off with dilute Giemsa's stain (e.g. Gurr's R66)–1 drop of stain to 1 cc. buffer solution at pH 7.0, and stain with this for a further 30 minutes. Wash with buffer solution. Blot and dry.

J. H. Wright's stain:

This is similar to Leishman's stain and is used more in America. It should be purchased ready for use. The method of staining is the same as for Leishman.

Giemsa's stain:

This consists of a number of compounds made by mixing different proportions of methylene blue and eosin. These have been designated Azur I, Azur II, and Azur II–eosin. The preparation can be purchased made up, but batches may vary considerably.

Lillie's method of preparing the stain:

(1) Azure B eosinate – Dissolve 10 g. of methylene blue in 600 cc. distilled water; add 6 cc. concentrated sulphuric acid. Bring to the boil and add 2.5 g. potassium bichromate dissloved in 25 cc. distilled water; boil for 20 minutes. Cool to 10°C or lower (place in refrigerator overnight). When cold, add 21 g. dry sodium bicarbonate slowly with frequent shaking. Then add a 5% solution of eosin (yellowish) and shake constantly until the margin of the fluid appears pale blue or bluish-pink. About 205 cc. will be required and 150 cc. of this can be added at once. Filter immediately, preferably on a vacuum funnel with hard paper. When the fluid

has been drawn through and the surface begins to crack, add 50 cc. distilled water. Allow to drain, and wash again with a second 50 cc. distilled water. Now wash with 40 cc. alcohol (95%) and repeat with a second 40 cc. alcohol. Dry the precipitate at room temperature or 37°C (not higher). This constitutes Azure B eosinate.

(2) Azure A eosinate - Proceed exactly as above, but use 5 g. potassium bichromate (in place of 2.5 g.) and dissolve it in 50 cc. distilled water.

(3) Methylene blue eosinate - Dissolve 10 g. methylene blue in 600 cc. cold distilled water and precipitate as before with 5% eosin solution, filtering and drying as above.

To make the finished stain, grind the three eosinates separately into fine powder in separate clean mortars. Then weigh out 500 mgm. azure B eosinate. 100 mgm. azure A eosinate, 400 mgm. methylene blue eosinate, and 200 mgm. finely ground methylene blue. Decant the mixed powder on to the surface of 200 cc. solvent, allowing it to settle gradually. Then shake frequently for two or three days, keeping the bottle between 50°C and 60°C between shakings. The solvent consists of equal volumes of methyl alcohol (A. R.) and glycerol (A. R.). The proportion of stains given above should yield a satisfactory staining picture. The diluting fluid is buffer solution pH. 7.0

This stain may be used in a manner somewhat similar to Leishman's preparation (the rapid method) or prolonged staining may be carried out as for

example, in staining spirochaetes (the slow method). In both cases the preparation must be fixed prior to staining, either by methyl alcohol for 3 minutes or by absolute for 15 minutes.

RAPID METHOD:

(i) Fix films in methyl alcohol – three minutes

(ii) Stain in a mixture of 1 part stain and 20 parts buffer solution pH. 7.0 for 1 hour.

(iii) Wash with buffer solution, allowing the preparation to differentiate for about a minute.

(iv) Blot and allow to dry in the air.

This method of staining gives excellent results with thin blood films for malarial parasites, Schuffner's dots being well defined. Trypanosomes are also well demonstrated.

A rapid method with the application of heat is useful for demonstrating spirochætes.

Fix preparations with absolute alcohol (15 minutes) or by drawing three times through a flame. Prepare a fresh solution of 10 drops of Giemsa solution with 10 cc. buffer solution of pH 7.0; shake gently and cover the fixed film with the diluted stain. Warm till steam rises, allow to cool for 15 seconds, then pour off and replace by fresh stain and heat again. Repeat the procedure four or five times, wash in distilled water, dry and mount.

SLOW METHOD:

This is a specially valuable method for demonstrating objects difficult to stain in the ordinary way,

e. g., certain pathogenic spirochætes. The principle is to allow the diluted stain to act for a considerable period. As the mixture of stain and water causes a fine precipitate, care has to be taken that this does not deposit on the film.

Cover-slips – The film is fixed in methyl alcohol for three minutes. A mixture is made in a Petri dish in the proportion of 1 cc. of stain to 2 cc. of buffer solution pH 7.0. The cover-slip, when fixed and still wet with the alcohol, is placed carefully film downwards on the surface of the mixture. When properly done, the coverslip remains floating. The lid is carefully placed on the Petri dish and the stain allowed to act overnight. The cover-slip is then washed in a stream of buffer solution, allowed to dry in the air, and mounted. There should be no deposit of precipitated stain on the preparation. Slides – the film is fixed in methyl alcohol for three minutes; the mixture of stain and buffer solution is made in a large (6-in) Petri dish if there are several slides to stain. A piece of thin glass rod is placed in the Petri dish, and the slides, after fixing, are laid film downwards in the fluid with one end of the slide resting on the glass rod so that there is sufficient staining fluid between the film and the bottom of the dish.

A sheet of glass slightly curved is also convenient; the slide with the film downwards, lies across the concavity, the space between containing diluted stain. The curved plate and slides should be placed in a box with a close fitting lid to avoid evaporation.

After 16 to 24 hours' staining, the slides are washed and dried as in the case of cover-slips. Adachi's modification – This method has been utilised for staining the flagella of Spirillum minus and can also be applied in the staining of delicate spirochætes. Fix the preparation for 30 to 60 seconds by *osmic acid vapour* over the following solution; osmic acid, 1 g; distilled water, 100 cc; 10 drops of 5% mercuric chloride; and then stain overnight in dilute Giemsa's solution to each 10 cc. of which 0.6 cc. of 1% potassium carbonate has been added.

Field's Rapid Method of Staining Thick Blood Film For Malarial Parasites.

This method can be recommended for routine use. In preparing the blood films it is important to ensure that they are *not too thick*. Drying may be assisted by placing the film in the incubator. After the film is quite dry, it may be passed very rapidly two or three times through a Bunsen or spirit flame, each passage occupying two or three seconds. When cool, the film is ready for staining.

Field's Stain.

(This stain in tablet form can be obtained from G. T. Gurr, London.)

Solution A (methylene blue) –

Methylene blue	1.3 g.
Disodium hydrogen phosphate (anhydrous)	5.0 g.
(If crystals, then 12.6 gr.)	

Dissolve in 50 cc. distilled water, bring to the boil and evaporate almost to dryness in a water bath, then add potassium dihydrogen phosphate (anhydrous 6.25 g.) if crystalline salt 8.0 g. Add 500 cc. of freshly boiled and still-warm distilled water, stir until the stain is completely dissolved and set aside for 24 hours. Filter before use. If a scum forms during use, filter again.

Alternately, if Azur I is available there is no need to carry out the polychroming of the methylene blue as outlined above, and solution A can be made as follows :-

Methylene blue	0.8 g.
Azur I	0.5 g.
Disodium hydrogen phosphate (anhydrous) (Crystalline salt 12.6 g.)	5.0 g.
Potassium dihydrogen phosphate (anhydrous) (crystalline salt g.)	6.25 g.
Distilled water	500 cc.

The phosphate salts are first dissolved in freshly boiled and still-warm distilled water and the stain is then added. Set aside for 24 hours and filter before use.

Solution B. (eosin)-

Eosin	1.3 g.
Disodium hydrogen phosphate (anhydrous) (crystalline salt 12.6 g.)	5.0 g.
Potassium dihydrogen phosphate (anhydrous) (crystalline salt 8.0 g.)	6.25 g.
Distilled water	500 cc.

The phosphate salts are first dissolved in freshly boiled and still-warm distilled water; then the stain is added. Set aside for 24 hours and filter before use.

The stains are kept in covered jars, the level being maintained by the addition of fresh stain as necessary. The same solution may be used continuously for many weeks without apparent deterioration, but the eosin solution should be renewed when it becomes greenish from the slight carry-over of methylene blue. If solutions show a growth of bacteria or moulds they should be discarded and replaced from stock solutions which, if stored carefully, will remain satisfactory upto a year.

Method of Staining:

(i) Dip the slide into the solution A for one to two seconds only.

(ii) Remove slide and immediately rinse gently in a jar of clean distilled or tap water until the stain ceases to flow from the film and the glass of the slide is free from stain.

(iii) Dip the slide into solution B for 1 to 2 seconds only.

(iv) Rinse gently for 2 to 3 seconds in clean water.

(v) Place vertically against a rack to drain and dry.

The relative times may require slight adjustment to suit different batches of stain. Films upto

three weeks old may benefit from immersion in phosphate buffer solution (as used for dissolving the stains) until hæmoglobin begins to diffuse out. The film is stained in the ordinary way.

Unduly thick films should be similarly immersed before staining to remove the greater part of the hæmoglobin. The phosphate buffer solution may be used in place of water for rinsing between solutions A and B.

SIMEONS' METHOD for staining THICK BLOOD FILMS for Malarial Parasites.

Solution I. (eosin solution)

Eosin pure	1.0 g.
Distilled water	1000 cc.

Solution II. (Stevenel's blue)

(a)	Medicinal methylene blue	1.0 g.
	Distilled water	75.0 cc.
	(dissolve completely)	
(b)	Potassium permanganate	1.5 g.
	Distilled water	75 0 cc.
	(dissolve completely)	

Mix (a) and (b) in a flask. A massive precipitate forms at once; keep the flask in a water-bath at boiling point for ½ hour during which the precipitate redissolves.

Filter. The stain is now ready for use: it requires no further dilution.

Procedure for staining thick films:

(i) Dehæmoglobinise by immersion in tap water in beaker No. 1, one minute.

(ii) Immerse in eosin solution, twenty seconds.

(iii) Rinse by dipping into tap water in beaker No. 2, four seconds.

(iv) Immerse in Stevenel's blue, thirty seconds.

(v) Rinse by dipping into tap water in beaker No. 3, four seconds.

(vi) Immerse again in eosin solution ten seconds.

(vii) Rinse by dipping into tap water in beaker No. 2, four seconds.

Blood Stains.

Giemsa Stain:

Azur II Eosin	3.0 g.
Azur II	0.8 g.
Glycerine, redistilled	250 cc.
Methyl alcohol, acetone free	250 cc.

Grind up the dyes in the alcohol heated at 60°C. Add glycerine heated at 60°C.

When all the dye is in solution, allow to stand at least 24 hours, then filter through filter paper. *The solution is stable.*

Wright Stain:

(a) Wright dye –

0.5% aqueous solution of sodium bicarbonate	100 cc.
Methylene blue	1 g.

Grind in a mortar until all methylene blue is in solution; place in Erlenmeyer flask to a depth of not more than 6 cm. Heat in an Arnold steam steriliser at 100°C for 1 hour. Cool, filter through filter paper. To every 100 cc. filtrate, add 500 cc. of a 0.1% aqueous solution of yellowish, water-soluble eosin. Add the eosin solution slowly, stirring constantly so as to form a precipitate. Test for a free eosin ring by placing a drop of the mixture on a piece of white filter paper and drying. The ring appears as a faint pink halo around a lavender spot. If the ring does not appear, add more 0.1% aqueous eosin until the ring forms. (In making Wright Dye with improved methylene blue we have found that about 700 to 850 cc. of 0.1% aqueous eosin solution is needed to produce an eosin halo). Filter through Whatmān 5 filter paper, discarding the filtrate. Save the precipitate on the filter paper. Dry the precipitate in the incubator; this may require several days. This is the stock stain. Place in a dark bottle, labelled "Wright Dye". This dye is stable.

(b) Wright Stain – Dissolve 0.1 gm. Wright dye, above, in 60 cc. methyl alcohol, acetone-free; the dye must be ground in a mortar and the alcohol added slowly until all the dye is in solution. Place in a dark bottle; allow to stand atleast two weeks before using.

Staining for Amoebae and Intestinal Protozoa in Faeces.

Stain I.

Alcohol, 1 part; saturated aqueous solution of ($HgCl_2$) Mercuric chloride, 2 parts. Stain for 5 minutes or longer.

Wash in 50% alcohol; put Gram's iodine; wash again with alcohol and then in water; then stain with No. II.

Stain II (a).

Mix equal parts of (a) and (b) before use.

(a)	Hematoxylin	1 g.
	Absolute alcohol	100 cc.

Stain II (b).

Liquor ferri perchlor (30%)	4 cc.
Concentrated hydrochloric acid	1 cc.
Distilled water	100 cc.

Wash in water; pass through alcohol; clear with xylol and counterstain with stain No. III.

Stain III.

Saturated aqueous solution of acid fuchsin	1 to 3 parts
Saturated aqueous solution of picric acid	100 parts

Dehydrate with absolute alcohol; clear in xylol and mount in balsam.

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